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(71) Applicant: GENZYME CORPORATION [US/US]; Legal Department, MetroWest Place, 15 Pleasant Street Connector, Framingham, MA 01701-9322 (US).

(72) Inventors: WADSWORTH, Samuel, C.; 10 Straw Hollow Lane, Shrewsbury, MA 01545 (US). O'RIORDAN, Catherine, R.; 16 Holbrook Lane, Jamaica Plain, MA 02130 (US). GOLDBERG, Mark; 41 Grasmere Road, Needham, MA 02494 (US).

(74) Agent: LAZAR, Steven, R.; Genzyme Corporation, Legal Department, 15 Pleasant Street Connector, Framingham, MA 01701-9322 (US).

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(54) Title: DRY POWDER COMPLEXES FOR GENE DELIVERY

(57) Abstract

The present invention is directed to dry powder formulations of a complex of a cationic molecule and an adenoviral vector which encodes a transgene of interest. In a preferred embodiment, the complex contains an adenoviral vector having at least one polyalkalene glycol polymer bound thereto. The invention also provides compositions containing these formulations in a physiologically acceptable carrier, methods for their preparation, and methods for their delivery to individuals.

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DRY POWDER COMPLEXES FOR GENE DELIVERY

BACKGROUND OF THE INVENTION

The effective treatment of inherited and acquired disorders is possible with the efficient delivery of transgenes which contain the coding sequences for proteins conferring specific phenotypic benefit to target cells of a recipient. Various vector systems, both viral and non-viral, have been developed that are capable of delivering a transgene to a target cell. While newer generations of vectors having improved characteristics have been developed, there still remains a need to improve efficiency of available gene transfer methods, and to provide gene delivery systems capable of generating the required level of transient or persistent gene expression. While some applications of gene delivery methods utilize persistent gene expression, other applications, the need for transient expression and/or repeat administration of a delivered transgene is indicated or preferred in other clinical applications. The present invention is directed to compositions containing adenoviral gene transfer vectors contained in dry powder formulations, and is also directed to methods for providing expression of a transgene by the administration of these compositions to individuals in need of such molecules.

Adenoviral vectors have been designed to take advantage of the desirable features of adenovirus which render it a suitable vehicle for nucleic acid transfer. Adenovirus is a non-enveloped, nuclear DNA virus with a genome of about 36 kb, which has been well-characterized through studies in classical genetics and molecular biology (Horwitz, M.S., "Adenoviridae and Their Replication," in Virology, 3rd edition, Fields et al., eds., Raven Press, New York, 1996). The viral genes are classified into early (known as E1-E4) and late (known as L1-L5) transcriptional units, referring to the generation of two temporal classes of viral proteins. The demarcation between these events is viral DNA replication. The human adenoviruses are divided into numerous serotypes (approximately 47, numbered accordingly and classified into 6 subgroups: A, B, C, D, E and F), based upon various properties including hemaglutination of red blood cells, oncogenicity, DNA base and protein amino acid compositions and homologies, and antigenic relationships.

Recombinant adenoviral vectors have several advantages for use as gene transfer vectors, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the potential to carry large inserts (Berkner, K.L., Curr. Top. Micro. Immunol. 158:39-66, 1992; Jolly, D.,

Cancer Gene Therapy 1:51-64, 1994).

The cloning capacity of an adenovirus vector is proportional to the size of the adenovirus genome present in the vector. For example, a cloning capacity of about 8 kb can be created from the deletion of certain regions of the virus genome dispensable for virus growth, *e.g.*, E3, and the deletion of a genomic region such as E1 whose function may be restored in trans from 293 cells (Graham, F.L., J. Gen. Virol. 36:59-72, 1977) or A549 cells (Imler et al., Gene Therapy 3:75-84, 1996). Such E1-deleted vectors are rendered replication-defective. The upper limit of vector DNA capacity for optimal carrying capacity is about 105%-108% of the length of the wild-type genome. Further adenovirus genomic modifications are possible in vector design using cell lines which supply other viral gene products in trans, *e.g.*, complementation of E2a (Zhou et al., J. Virol. 70:7030-7038, 1996), complementation of E4 (Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995; Wang et al., Gene Ther. 2:775-783, 1995), or complementation of protein IX (Caravokyri et al., J. Virol. 69:6627-6633, 1995; Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995).

Adenoviral vectors for use in gene transfer to cells and in gene therapy applications commonly are derived from adenoviruses by deletion of the E1 region of the adenoviral genome (Berkner, K.L., Curr. Top. Micro. Immunol. 158:39-66, 1992). Deletion of E1 renders the vector replication defective and significantly reduces expression of the remaining viral genes present within the vector. However, it is believed that the presence of the remaining viral genes in adenovirus vectors can be deleterious to the transfected cell for one or more of the following reasons: (1) stimulation of a cellular immune response directed against expressed viral proteins, (2) cytotoxicity of expressed viral proteins, and (3) replication of the vector genome leading to cell death.

Transgenes that have been expressed to date by adenoviral vectors include p53 (Wills et al., Human Gene Therapy 5:1079-188, 1994); dystrophin (Vincent et al., Nature Genetics 5:130-134, 1993; erythropoietin (Descamps et al., Human Gene Therapy 5:979-985, 1994; ornithine transcarbamylase (Stratford-Perricaudet et al., Human Gene Therapy 1:241-256, 1990; We et al., J. Biol. Chem. 271;3639-3646, 1996;); adenosine deaminase (Mitani et al., Human Gene Therapy 5:941-948, 1994); interleukin-2 (Haddada et al., Human Gene Therapy 4:703-711, 1993); and α1-antitrypsin (Jaffe et al., Nature Genetics 1:372-378, 1992); thrombopoietin (Ohwada et al., Blood 88:778-784, 1996); and cytosine deaminase (Ohwada et al., Hum. Gene Ther. 7:1567-1576, 1996).

The tropism of adenoviruses for cells of the respiratory tract has particular relevance to the use of adenovirus as a gene transfer vector for cystic fibrosis (CF), which is the most common autosomal recessive disease in Caucasians. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that disturb the cAMP-regulated Cl^- channel in airway epithelia result in pulmonary dysfunction (Zabner et al., Nature Genetics 6:75-83, 1994). Adenoviral vectors engineered to carry the CFTR gene have been developed (Rich et al., Human Gene Therapy 4:461-476, 1993) and studies have shown the ability of these vectors to deliver CFTR to nasal epithelia of CF patients (Zabner et al., Cell 75:207-216, 1993), the airway epithelia of cotton rats and primates (Zabner et al., Nature Genetics 6:75-83, 1994), and the respiratory epithelium of CF patients (Crystal et al., Nature Genetics 8:42-51, 1994). Transfer of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to airway epithelia of patients with cystic fibrosis (CF) provides an example of successful use of gene transfer to correct a cellular defect, *i.e.*, the CF defect in electrolyte transport. Vector systems using adenoviral vectors (Zabner et al., Cell 75: 207, 1993; Knowles et al., New Engl. J. Med. 333: 823, 1995; Hay et al., Hum. Gene. Ther. 6: 1487, 1995; Zabner et al., J. Clin. Invest. 97: 1504, 1996 and U.S. Patent No. 5,670,488) and/or cationic lipids (Caplen et al., Nat. Med. 1: 39, 1995; U.S. Patent No. 5,650,096) have been shown to be capable of transferring the CFTR cDNA and expressing CFTR in mature ciliated human airway epithelia. The successful delivery of CFTR in such cells is evidenced by the establishment of a functional chloride ion channel in the treated cells.

Other gene transfer systems that combine viral and nonviral components have been reported (Cristiano et al., Proc. Natl. Acad. Sci. USA 90: 11548, 1993; Wu et al., J. Biol. Chem. 269: 11542, 1994; Wagner et al., Proc. Natl. Acad. Sci. USA 89: 6099, 1992; Yoshimura et al., J. Biol. Chem. 268: 2300, 1993; Curiel et al., Proc. Natl. Acad. Sci. USA 88: 8850, 1991; Kupfer et al., Hum. Gene Ther. 5: 1437, 1994; and Gottschalk et al., Gene Ther. 1: 185, 1994). In most cases, adenovirus has been incorporated into the gene delivery systems in order to take advantage of its endosomolytic properties. The reported combinations of viral and nonviral components generally involve either covalent attachment of the adenovirus to a gene delivery complex or co-internalization of unbound adenovirus with cationic lipid: DNA complexes. Further, the transferred gene is contained in plasmid DNA that is exogenous to the adenovirus. In these formulations, large amounts of adenovirus are required, and the increases in gene transfer are often modest (Cotten et al., Proc. Natl. Acad. Sci. USA 89:6094-6098,

1992; Wagner et al., Proc. Natl. Acad. Sci. USA 89:6099-6103, 1992; Schwarzenberger et al., J. Virol. 71:8563-8571, 1997; Fasbender et al., J. Clin. Invest. 102:184-193, 1998).

It has been demonstrated that nucleic acids can be delivered to the lungs by different routes, including intratracheal administration of a liquid suspension of the nucleic acids and inhalation of an aqueous aerosol mist produced by a liquid nebulizer or the use of a dry powder apparatus such as that described in U.S. Patent No. 5,780,014, the disclosure of which is incorporated by reference. Transfer of an adenoviral vector containing the CFTR transgene in animal studies has been generally been accomplished by intranasal installation (Armentano et al. J.Virol. 71:2408-2416, 1997; Kaplan et al., Human Gene Therapy 9:1469-1479, 1998), although aerosol administration by inhalation to a non-human primate resulted in the expression and delivery of the CFTR transgene (McDonald et al., Human Gene Therapy 8:411-422, 1997).

Intratracheal administration is not suitable for routine therapeutic use in humans and has a very low patient acceptability. Moreover, intratracheal instillation often results in very uneven distribution of a dispersion in the lungs, with some regions receiving very little or no material. The use of a liquid nebulizer enjoys higher patient acceptability and achieves better distribution, but requires time-consuming equipment set-up, can require prolonged periods of treatment to achieve an adequate dosage, can inactivate a viral carrier, and can result in undesirable aggregation or degradation of the nucleic acids within the aerosol mist. Aggregated nucleic acids will generally be less suitable for uptake into host target cells. Transgene delivery is aided, therefore, by the preparation of compositions which are refractory to such aggregation. For example, methods to formulate polynucleotide complexes into dry powder compositions have been described in U.S. Patent No. 5,811,406, the disclosure of which is incorporated by reference.

Accordingly, it would be desirable to provide aerosolized dry powder adenoviral vector compositions, methods for making such compositions, and methods for their delivery to individuals in need of such vectors as a means of improving the efficiency of adenoviral vector transgene delivery. The present invention is directed to providing such compositions and methods.

SUMMARY OF THE INVENTION

The present invention is directed to dry powder formulations of a complex of a cationic molecule and of an adenoviral vector. The adenovirus component of the complex is preferably a recombinant adenoviral vector which contains a transgene of interest, such as

CFTR. In a preferred embodiment, the adenoviral vector has at least one polyalkalene glycol polymer bound thereto, and the polyalkalene glycol polymer is preferably polyethylene glycol (PEG). The cationic molecule component of the complexes of the present invention can be a cationic polymer, with DEAE-Dextran being preferred. Alternatively, the cationic molecule is a cationic lipid.

The invention also provides compositions containing the dry powder formulations of the invention in a physiologically acceptable carrier, methods for their preparation, and methods for their delivery to individuals.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to dry powder formulations of complexes between cationic molecules and polymer-modified adenovirus that advantageously exhibit increased infectivity and reduced immunogenicity. These adenoviral vector complexes of the present invention have surprisingly been found to exhibit heightened levels of infectivity in cells previously immunized with adenovirus, in addition to heightened levels of infectivity in naive (*i.e.*, non-immunized) cells. They are particularly advantageous for ease of transgene delivery to individuals in need of such molecules.

The dry powder compositions of the invention can be readily dispersed in a flowing gas stream to provide a dry aerosol for delivery to an individual. The advantages of the compositions of the invention include providing the delivery of required dosages of nucleic acids in a very rapid manner (typically in several or fewer breaths), convenience of administration, and the capacity of such compositions to be stored over extended periods. The dry powders are delivered to particular target regions within the host and are readily dispersed over the internal surfaces of the target tissue, preferably the lung, where the powder rehydrates rapidly in the moist layer over the surfaces to release nucleic acids for delivery into the target cells.

In accordance with the present invention, adenoviral vectors are polymer-modified by covalently or noncovalently binding to the virus a polyalkalene glycol polymer, which renders the viral vector substantially non-immunogenic. Reduced immunogenicity of an adenoviral vector is therefore an advantage of the present invention. The polyaklene glycol polymers used in the present invention preferably have an average molecular weight of from about 200 to about 20,000 daltons. Examples of glycol polymers that can be used include, but are not limited to, polyoxymethylene glycols, polyethylene glycols (PEG), methoxypolyethylene

glycols, and derivatives thereof including for example polymethyl-ethylene glycol, polyhydroxypropylene glycol, polypropylene glycol, and polymethylpropylene glycol.

In another embodiment of the present invention, the polyalkalene glycol poymer is an activated polyalkylene glycol polymer. Examples of activated polyalkylene glycol polymer that can be used include, but are not limited to, methoxypolyethylene glycol-tresylate (TMPEG), methoxypolyethylene glycol-acetaldehyde, methoxypolyethylene glycol activated with cyanuric chloride, N-hydroxysuccinimide polyethylene glycol (NHS-PEG), polyethyleneglycol-N-succimimide carbonate and mixtures thereof.

A preferred glycol polymer used in accordance with the present invention is PEG. PEG is a water-soluble polymer having the formula $H(OCH_2CH_2)_nOH$, wherein n is the number of repeating units and determines the average molecular weight. PEGs having average molecular weights of from 200 to 20,000 daltons are commercially available from a variety of sources. In accordance with the present invention, PEG having an average molecular weight of from 200 (PEG_{200}) to 20,000 ($PEG_{20,000}$)can be used to prepare adenoviruses modified with PEG. In a preferred embodiment, PEG has an average molecular weight from about 2000 to about 12,000, with an average molecular weight from about 4000 to about 6000 (*e.g.*, 5000) being more preferred.

In accordance with the present invention, the polyalkalene glycol polymer is directly covalently bound to the virus particle, indirectly covalently bound to the virus particle by an intermediate coupling moiety, directly noncovalently attached to the virus particle, or indirectly noncovalently attached to the virus particle by a ligand.

A variety of schemes exist for covalent and non-covalent attachment: 1) the glycol polymer can be attached via direct covalent coupling to the surface of the adenovirus; 2) the glycol polymer can be attached via indirect covalent coupling (*e.g.*, via an intermediate coupling moiety that links the polymer to the adenovirus surface); or 3) the glycol polymer can be attached via an indirect non-covalent linkage using, for example, a suitable PEGylated ligand. The ligand for indirect noncovalent attachment is preferably a ligand having specificity for a viral surface component, such as an antibody. One particularly preferred antibody to be used is a non-neutralizing anti-adenovirus antibody, such as a non-neutralizing anti-hexon antibody. Examples of suitable ligands include, but are not limited to, antibodies to surface proteins, lipids or carbohydrates.

Targets for polymer modification include reactive groups on the viral surface with which the polymer or coupling agent can interact, including for example primary and secondary

amine groups, thiol groups and aromatic hydroxy groups. As will be apparent to one of ordinary skill in the art, the preferred method for polymer modification of the adenovirus is dependent upon the available target sites found on the viral surface. Examples of available target sites for attachment of the glycol polymer to the adenovirus include, but are not limited to, the hexon, penton cell base, and fiber proteins. The adenoviral hexon protein is a particularly preferred site for attachment of the alkalene glycol polymer. While not wishing to be bound by theory, it is believed that modification of these sites masks epitopes from neutralizing antibodies, thereby providing the adenoviral vector with reduced antigenicity and/or immunogenicity, allowing for more persistent expression of a transgene and for repeat administration of the adenoviral vector.

Methods for the direct or indirect covalent attachment of polymers to polypeptides that are known in the art may be used to provide the polymer-modified adenoviruses of the present invention. Methods are described, for example, in WO90/04606, and in U.S. Patent Nos. 4,179,337 and 5,612,460, the disclosures of which are incorporated herein by reference. Generally, the glycol polymer is activated by converting a terminal moiety of the polymer to an activated moiety, or by attaching an activated coupling moiety to the polymer. The activated polymer is then coupled to the target via the activated moiety. The activated moiety or activated coupling moiety can be selected based upon its affinity for the desired target site on the viral surface.

For example, the terminal hydroxyl groups of PEG can be converted into reactive functional group or attached to an activated coupling moiety to provide a molecule known as "activated" PEG. Various forms of activated PEG are known in the art and are commercially available. For direct covalent linkage to the adenovirus a suitable activated PEG is MPEG-tresylate (TMPEG), which is believed to react with ε-lysine groups, or MPEG-acetaldehyde. For indirect covalent linkage other forms of activated PEG are known in the art and commercially available, including for example methoxypolyethylene glycol (MPEG) derivatives such as MPEG activated with cyanuric chloride, PEG N-hydroxysuccinimide PEG (NHS-PEG), which reacts with amine groups, and PEG-N-succimimide carbonate. These and other activated PEGs are disclosed in W095/06058, and in U.S. Patent Nos. 4,179,337 and 5,612,460, which are incorporated herein by reference.

The covalent attachment of PEG to the adenovirus surface ("PEGylation") is accomplished by incubating the virus with the activated PEG (*e.g.*, TMPEG). Single addition or multiple addition incubation regimes can be used. The optimal ratios of TMPEG to

adenoviral particles to achieve reduced antigenicity, along with heightened infectivity, may be ascertained by performing the various assays described below. Under conditions designed to provide direct TMPEG modified adenovirus, PEGylation in the amount of about 5-20% w/v is preferred, with a concentration of about 10% w/v being most preferred.

Preferably, when high concentrations (*e.g.*, 10% or greater) of glycol polymer are attached to the virus, the activated polymer is added in a stepwise fashion. Stepwise addition is preferred since viral particles tend to aggregate at high concentrations, which reduce the overall effeciency of PEGylation. Moreover, aggregation is exacerbated by the use of certain activated polymers, *e.g.*, TMPEG. Thus, the inital use of low polymer concentrations in a stepwise manner can reduce the tendency of the particles to aggregate, thereby facilitating a higher degree of PEGylation. For example, activated PEG such as TMPEG may be added in separate steps to a viral stock solution every thirty minutes to increase the polymer concentration each time by 3%, 5% or 8% (w/v) in the reaction mixture to obtain final polymer concentrations of 12%, 20% and 32% respectively (approximately w/v, *i.e.*, not correcting for the volume of the polymer). In addition, after the last addition of the glycol polymer, a further incubation time might be allowed. These necessary adjustments to the reaction parameters (*e.g.*, the number of steps, concentration of the polymer, and reaction time) for optimal results can easily be ascertained by one of ordinary skill in the art.

The attachment reaction may be quenched by dialysis or by addition of excess lysine (*e.g.*, a 10 to100-fold excess lysine). Alternatively, the reaction might be run to completion (*i.e.*, the point at which the activated PEG, such as TMPEG, is either completely consumed in the PEGylation reaction or rendered inactive by hydrolysis).

In another embodiment of the present invention, the glycol polymer is indirectly noncovalently attached to the adenovirus via a suitable ligand. In a preferred embodiment, the ligand is an antibody or antibody fragment, including for example a non-neutralizing anti-virus antibody or fragment therefrom (*e.g.*, Fab, $F(ab')_2$, Fv). As used herein, the term "antibody" includes monoclonal and polyclonal antibodies. In a particularly preferred embodiment, the ligand is a non-neutralizing anti-hexon antibody. Such antibodies are commercially available and include, for example, MAb 8052 and MAb 805 available from Chemicon International, Temecula, CA, USA.

Indirect non-covalent attachment of glycol polymer to the adenovirus is accomplished by incubation of the virus with a suitable ligand that has been modified by the covalent attachment of polymer. The glycol polymer can be covalently attached (*i.e.*, bound) to the

ligand by standard methods as described herein above. For example, a non-neutralizing anti-virus antibody such as anti-hexon antibody may be PEGylated using an activated PEG molecule as described above. In a preferred embodiment, anti-hexon antibody is modified using TMPEG. One of ordinarly skilled in the art can ascertain the optimal ratios of activated PEG to antibody, concentrations of activated PEG and antibody, buffer and time and temperature of incubation to achieve optimal modification of the antibody. The polymer-modified ligand is then incubated with adenovirus to allow non-covalent binding of the polymer-modified ligand to the virus surface.

Antibodies modified with PEG at the epitope binding site (*e.g.*, complementarity determining regions (CDRs) can exhibit reduced affinity to the adenovirus thereby decreasing the efficiency of noncovalent attachment. In order to prevent PEGylation at the epitope binding site an antibody is preferably immobilized prior to PEG modification. For example, anti-hexon antibody is bound to purified immobilized hexon (*e.g.*, hexon-Sepharose) prior to PEG modification of antibody. The PEGylated antibody is then released from immobilized hexon.

Alternatively, non-immobilized anti-hexon antibodies can be PEGylated creating a population of antibodies PEGylated on the epitope binding site in addition to other sites, which are thereafter separated by immunoaffinity chromatography. For example, the mixed population of modified antibodies can be incubated with immobilized hexon, to which antibodies modified only at sites other than the epitope binding site will bind. These PEGylated antibodies are then released from the immobilized hexon for use in accordance with the present invention.

For some applications, for example, those requiring repeat dosing of a polymer modified virus, it may be desirable to separate the unreacted glycol polymer from the polymer-modified adenovirus, which may then be purified by standard methods as necessary for the intended use. Separation and purification may be performed by methods known in the art, for example ion exchange chromatography, gel filtration chromatography, or cesium chloride gradient purification. In situations in which there is indirect PEGylation of an antibody, hexon affinity resin may be useful to separate the PEGylated antibody from unreacted PEG.

In addition, it may be desirable to separate unmodified adenovirus from polymer-modified adenovirus. Separation of the unmodified from polymer-modified virus may be performed by partitioning in an aqueous biphasic polyalkylene glycol solution. For example, phase partitioning in an aqueous biphasic system of PEG and dextran may allow the separation of PEG-modified virus from unmodified virus. Partitioning may be performed by counter-

current distribution. Generally, the phase system is prepared by mixing solutions of dextran and PEG. PEG and PEG-modified virus are incorporated into the phase system, mixed by inversion or rotation, and allowed to separate. PEG modified virus partitions into the PEG phase, and unmodified virus partitions into the dextran phase.

The efficiency of adenovirus polymer modification (*e.g.*, PEGylation) is evaluated by methods known in the art, including ion exchange chromatography, capillary electrophoresis (CE), photon correlation spectroscopy (PCS), and through the use of a labeled (*e.g.*, biotinylated) PEG in a quantitative ELISA. Ion exchange chromatography (*e.g.*, DEAE-chromatography) can be performed by standard methods to evaluate the modified viruses based upon altered charge.

Whole virus CE provides a means to monitor the modification of adenovirus by the glycol polymer as a function of altered surface charge. For example, covalent attachment of PEG to the adenovirus surface seems to result in shrouding of the negative surface charges on the viral particle thereby causing virus to exhibit a more neutral mobility. CE may be performed by methods known to those of ordinary skill in the art. For instance, a ramped low-high voltage pre-treatment is used to electrophorese the highly mobile salt ions in which the virus may be formulated for stability, before true, high voltage separation begins. In plots derived from CE, virus particles with PEG covalently attached run at a position closer to the neutral point than virus without covalently attached PEG. CE may be conveniently used to assess the influence of various conditions, including molar ratios, concentrations and incubation times, on the covalent attachment of PEG to the virus particles. Increasing neutrality reflects increasing PEG-chain density on the virus surface.

PCS uses the relationship between particle size and movement in suspension (via Brownian motion) to gain accurate measurements on the size of the particles. This method is widely applied to monitor polymer attachment to particles including liposomes, microspheres and nanoparticles by measuring their increase in size. These data suggest that covalently attached PEG at relatively low density forms globular "mushroom" shapes and thus the increase in size is relatively small. Altering the conditions under which one would expect to increase the density of covalently attached PEG chains results in a more extended conformation of the polymer or "brush" shapes which is reflected by a relatively larger increase in particle size. Thus PCS may be used using methods known to those of ordinary skill in the art to monitor the size changes of the virus particle under different reaction conditions.

The ELISA analysis of a biotinylated PEG can provide the most quantitative assessment of the number of molecules of PEG covalently bound to the adenovirus virus particle. The ELISA can be performed by standard methods known in the art.

The indirect noncovalent attachment of glycol polymer via a polymer-modified ligand can also be monitored by displacement of labeled ligand from the adenovirus in a competition enzyme-linked immunosorbent assay (ELISA). For example, the ability of a PEGylated anti-hexon antibody to bind to the adenovirus surface is measured in a standard competition ELISA using a biotinylated anti-hexon antibody.

The adenovirus component of a complex of the invention can be any adenovirus, but preferably a recombinant adenoviral vector which contains a transgene of interest. Preferably, the recombinant adenovirus is also engineered to be incapable of replicating and exhibits minimal expression of adenoviral genes in order to minimize the generation of a host immune response. Suitable recombinant adenoviral vectors are preferably derived from, but not limited to, adenovirus serotypes type 2 (Ad2), type 5 (Ad5), and type 17 (Ad17). Preferably, the recombinant adenoviral vectors have been deleted for the E1 region, which renders such vectors replication-defective and which allows for the insertion of one or more transgenes into this site. Such vectors can be produced using cell lines which complement the E1 deletion, for example, the 293 cell line (Graham et al., J. Gen.Virol. 36:59-72, 1977), the disclosure of which is incorporated by reference.

Representative adenoviral vectors that are useful for delivery of a transgene and which can be incorporated into the dry powder compositions of the invention are disclosed by Zabner et al., Cell 75 : 207, 1993; Zabner et al., J. Clin. Invest. 6 : 1504, 1996; Armentano et al., J. Virol. 71:2408-2416, 1997; Scaria et al., J. Virol. 72:7302, 1998; Kaplan et al., Human Gene Ther. 9:1469-1479, 1998; and U.S. Patent Nos. 5,707,618 5,670,488 and 5,824,544, the disclosures of which are incorporated herein by reference. The recombinant adenoviral vectors used in the complexes of the invention may have any genomic configuration, including, but not limited to, the inclusion or deletion of all or part of the E1, E2, E3, E4 genomic regions and any or all of the late gene regions of the adenoviral genome. The vectors of the complexes may also have wild-type or altered structural proteins, including fiber, penton and hexon, or may have chimeric surface proteins which are derived from one or more adenovirus serotypes.

The recombinant adenoviruses also preferably contain transgenes operably linked to suitable promoter and other expression control sequences. A gene is defined as a nucleotide sequence which encodes an mRNA, antisense nucleic acid, ribozyme, protein, or other

biologically relevant molecule. A transgene is defined as a gene that is not native to the adenovirus genome, i.e., heterologous. Examples of transgenes to be utilized in the invention are nucleic acids encoding a biologically functional protein or peptide, an antisense molecule, ribozyme, or marker molecule. The promoter may be an endogenous adenovirus promoter, for example the E1a promoter or the Ad2 major late promoter (MLP) or a heterologous eucaryotic promoter, for example a phosphoglycerate kinase (PGK) promoter or a cytomegalovirus (CMV) promoter. Similarly, those of ordinary skill in the art can construct adenoviral vectors utilizing endogenous or heterologous polyA addition signals.

In one preferred embodiment of the present invention, an adenovirus complex contains a transgene encoding erythropoetin (EPO) (U.S. Patent No. 4,703,008, the disclosure of which is incorporated by reference). Preferably, the transgene, comprising either the wild-type gene or the cDNA encoding EPO, is inserted into an E1-deleted adenovirus. This embodiment of the invention further provides for the expression of the EPO transgene using expression control sequences, including promoters. Such sequences may be chosen in order to preferentially select a level of expression of the transgene, for example, using a CMV promoter to obtain high expression levels, a PGK promoter to obtain moderate expression levels, or an adenovirus E1A promoter to achieve lower expression levels. The use of other expression control sequences in the adenoviral vector containing the EPO-encoding DNA sequence is also within the scope of the invention, including other promoter , enhancer or polyadenylation elements. Dry powder formulations of adenoviral vector complexes containing the EPO transgene are advantageous as they are readily administered and can provide transient expression of EPO for such clinical indications as increasing pre-surgical hematocrit levels or the treatment of anemia. The transience of expression and the reduced immunogenicity of these formulations supports repeated administration of the compositions and allows for the administration of a series of short courses of treatment.

In other preferred embodiments of the invention, the transgene encoded by a recombinant adenoviral vector is granulocyte colony stimulating factor (G-CSF) (U.S. Patent No. 4,999,291), granulocyte-macrophage colony stimulating factor (GM-CSF) (U.S. Patent No. 5,602,007) or interferon-alpha (Houghton et al., Nature 285:136, 1980; Diaz et al., J. Interferon Res. 14:221, 1994), the disclosures of which are incorporated by reference.

In another preferred embodiment of the invention, the recombinant adenoviral vector contains a transgene encoding cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a phosphorylation and nucleoside triphosphate-regulated Cl^- channel located in the

apical membrane of epithelial cells in the lung, intestine, pancreas and sweat glands (Welsh et al., Neuron 8: 821, 1992), the disclosure of which is incorporated by reference. Cystic fibrosis (CF) results from a non-functional Cl^- channel in an individual's epithelial cells caused by mutations in the gene encoding CFTR. Such mutations result in loss of function of the chloride channel and thus defective electrolyte transport in affected epithelial cells. DNA encoding wild-type CFTR is known in the art; the sequence is disclosed, for example, in U.S. Patent No. 5,670,488, incorporated herein by reference. A deletion mutant of CFTR that encodes a regulated Cl^- channel is disclosed by Sheppard et al. (Cell 76: 1091, 1994) and in U.S. Patent No. 5,639,661, the disclosures of which are incorporated herein by reference. Other examples of recombinant adenoviral vectors containing transgenes encoding CFTR are Ad2/CFTR-2, Ad2/CFTR-8, and Ad2/CFTR-16, which are respectively found in U.S. Patent No. 5,670,488, U.S. Patent No. 5,707,618 and Scaria et al. (J. Virol. 72:7302, 1998), all incorporated herein by reference.

In accordance with the present invention, DNA encoding a CFTR protein includes the foregoing published sequences as well as other DNAs encoding CFTR known to those of skill in the art. Further included are modifications of the known DNA sequence encoding CFTR, for example mutations, substitutions, deletions, insertions and homologs, that encode a functional CFTR protein, *i.e.*, a chloride channel.

In another embodiment of the invention, the polymer-modified recombinant adenovirus is an adenovirus that can induce tumor-specific cytolysis also known as viral oncolysis. Representative adenovirus that are useful for viral oncolysis are disclosed by Bischoff et al., Science 274:373, 1996, and Heise et al., Nature Medicine 3:630, 1997 and EP689447A, the disclosures of which are incorporated herein by reference.

In accordance with the present invention, the polymer-modified adenovirus described above is further complexed with a cationic molecule and formulated into a dry powder composition. The cationic molecule can be any cationic compound that exhibits minimal toxicity to mammals and does not decrease the infectivity of the polymer-modified virus. Preferably, the cationic molecule provides the polymer-modified virus with infectivity levels comparable to, if not greater than, the infectivity levels exhibited by the corresponding unmodified adenovirus. Examples of cationic molecules that can be used include, but are not limited to, cationic polymers, cationic lipids, cationic sugars, cationic proteins, or cationic dendrimers. The cationic molecules can also be combined with non-cationic molecules. Examples of cationic polymers include, but are not limited to, polyethyleneimine (PEI), DEAE-

dextran, and histone (fraction V-S), protamine, poly-L-lysine (PLL), polybrene (Hexadimethrine Bromide) and cationic dendrimers, in which DEAE-dextran is preferred. Alternatively, the polymer-modified adenovirus can be dispersed in a metal salt precipitate such as calcium phosphate. Those of ordinary skill in the art can determine the molecular weight of the cationic polymer that provides optimal gene transfer in accordance with the methods described herein. PEI is preferably used at an average molecular weight of 25 kDa.

Cationic lipids are known to those of ordinary skill in the art. Representative cationic lipids include those disclosed *e.g.*, by U.S. Patent No. 5,283,185, WO96/18372, and U.S. Patent No. 5,650,096, the disclosures of which are incorporated herein by reference. In a preferred embodiment the cationic lipid is (N-(N^1,N^1-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) disclosed in U.S. Patent No. 5,283,165. In another preferred embodiment, the cationic lipid is N^4-spermine cholesterol carbamate (GL-67) or N^4-spermidine cholesterol carbamate (GL-53) disclosed in WO96/18372 and U.S. Patent No. 5,650,096. Other representative cationic lipids include (2, 3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), commercially available as TRANSFECTAM® from Promega, Madison, WI; 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propyl amide (DOSPER); N-[1-(2,3-Dioleoyloxy)propyl] -N,N,N-trimethyl-ammoniummethylsulfate (DOTAP); N-[1-2(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA); (±)-N-(2-Hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE); (±)-N-(2-Aminoethyl)-N,N-dimethyl-2,3-bis (tetradecyloxy)-1- propanaminium bromide (βAE-DMRIE); dimethyldioctadecylammonium bromide (DDAB); LIPOFECTIN®, a 1:1 (w/w) formulation of DOTMA and dioleoyl phosphotidylethanolamine (DOPE) commercially available from Life Technolgies, Gaithersburg, MD; LIPOFECTAMINE®, a 3:1 (w/w) formulation of DOSPA and DOPE commercially available from Life Technologies, Gaithersburg, MD; LIPOFECTACE™, a 1:2.5 (w/w) formulation of DDAB and DOPE, commercially available from Life Technologies, Gaithersburg, MD; Tfx™ -50, a reagent consisting of N,N,N', N'-tetramethyl-N-N'-bis(2-hydroxyethyl)-2,3,-dioleoyloxy-1, 4-butanediammonium iodide and DOPE, commercially available from Promega, Madison, WI; and DMRIE-C™, a 1:1 (molar ratio) formulation of DMRIE and cholesterol commercially available from Life Technologies, Gaithersburg, MD. In preferred embodiments the cationic lipid is GL-53 or GL-67. In accordance with the present invention, the cationic lipid may be combined with a colipid such as DOPE or cholesterol in the formation of the adenoviral complexes of the invention.

The ratio of cationic molecule to polymer-modified adenovirus to be used in complex formation is variable. As will be apparent to one skilled in the art, factors that may affect the virus:cationic molecule ratios include the cationic molecule selected, polyalkylene glycol polymer selected, and cell type targeted for infection. However, optimal virus:cationic molecule ratios can easily be determined by one skilled in the art utilizing the infectivity assays described herein to determine those ratios that yield optimal expression of a transgene. Some illustrative cationic molecule:virus ratios for the complexes of the present invention are set forth below. DEAE-Dextran is complexed with the polymer-modified adenovirus at a ratio of 100-3000 molecules per virus particle, with 400-600 molecules per virus particle being preferred. In the formation of dry powder compositions, preferred ratios may be from 100,000 to 500,000 molecules per virus particle. PEI is complexed at a ratio of 200-1200 molecules per virus particle, with 400-600 molecules per virus particle being preferred. Protamine is complexed with 400-40,000 molecules per virus particle, with 3000-5000 molecules per virus particle being preferred for an aerosolized composition. Polybrene is complexed with 4×10^3 - 4×10^5 molecules per virus particle, with 3.5×10^5 - 4.5×10^5 molecules per virus particle being preferred. The cationic lipid, GL-67, is complexed with 9×10^5 - 9×10^6 molecules per virus particle, with 8.5×10^6 - 9.5×10^6 molecules per virus particle being preferred. Alternatively, calcium phosphate can be co-precipitated in the presence of the polymer-modified virus by admixing a molar excess of calcium (Ca^{2+}) to phosphate (PO_4^-), ($Ca^{2+} : PO_4^-$), ranging from 6:1 to 42:1, with a ratio 13:1 to 15:1 being preferred, or with the use of a 0.5X Ca++ solution applied to the target cells.

The complexes of the dry powder formulations of the present invention can be simply prepared by admixing the components under suitable conditions. For example, suspensions of viral particles and cationic molecules are prepared with phosphate-buffered saline (PBS) at pH of 7. Prior to admixing the two suspension, the suspensions are warmed to 30°C to facilitate complex formation. The two suspensions are mixed and incubated at 30°C for approximately 15 minutes to allow sufficient complex formation. However, if desired, complex formation can be conducted at room temperature with additional incubation times. The complexed polymer-modified adenovirus is then resuspended in PBS and is available for processing into a dry powder formulation.

The present invention is directed to compositions which are dry powder formulations of the adenoviral vector complexes described above. These compositions can be readily dispersed in a flowing gas stream to provide aerosol delivery of the adenoviral vector

complexes to an individual, and can be easily readministered, particularly in clinical situations in which periodic transient gene expression is desirable. The dry powder formulations will permit delivery of required dosages of nucleic acids in a very rapid manner (typically in several or fewer breaths) and will be suitable for storage over extended periods. The dry powders are delivered to particular target regions within the host and are readily dispersed over the internal surfaces of the tissue such as the lung, where the powder or aerosol reconstitutes or rehydrates rapidly in the moist layer over the surfaces to thereby release nucleic acids to interact with the target cells. Methods for making such powders or aerosols will preferably provide compositions having suitable characteristics for pulmonary delivery without the need for size reduction, aggregation, mixing, size selection, and/or other steps.

The dry powder compositions of the invention comprise nucleic acids, whether in adenoviral vectors, plasmids or other forms, dispersed or encapsulated within a matrix of hydrophilic excipient material. Preferably, the nucleic acid is an adenoviral vector complex or a nucleic acid construct. Preferred adenoviral vector complexes of the present invention are defined as adenoviral vectors which are polymer-modified by covalently or noncovalently binding to the virus a polyalkalene glycol polymer, which renders the viral vector substantially non-immunogenic and further containing a cationic molecule (e.g., DEAE-dextran) or cationic lipid (e.g., DOTMA, GL-67) or lipid-containing material, optionally but not necessarily in the form of a liposome or other vesicle, thereby creating a complex of the invention.

A nucleic acid construct of the present invention is defined as nucleic acids which are present as bare nucleic acid molecules, viral vectors, associated viral particles, plasmid DNA or RNA, or other nucleic acid constructions of a type suitable for gene transfer by incorporation into a dry powder composition of the invention.

Preparation of the dry powder compositions of the invention can be performed by standard techniques, including the use of an excipient. The excipient to be used in formulating a dry powder composition of the invention will be selected based on a number of criteria, including physiological acceptability, ability to protect the nucleic acid during preparation of the powder, the ability to preserve the nucleic acid during storage and prior to use, and the ability to provide certain desirable powder characteristics depending on the intended use. By "physiological acceptability," it is meant that the excipient will be suitable for pulmonary delivery to a human or other host without deleterious effects, i.e., that the excipient will be biocompatible. The excipient will preferably protect the nucleic acid during all phases of the production process, particularly during spray-drying procedures where an aqueous

solution of nucleic acid and the excipient(s) will be exposed to relatively high temperatures, shear forces, and other harsh conditions. The excipient will also be able to act as a stabilizer for the nucleic acids to prevent degradation after production when the nucleic acid compositions are stored, usually at non-refrigerated room temperatures, over extended periods of time. The excipient will also provide bulk in the formulation of compositions where the nucleic acid concentration is low, i.e., a bulking agent. Finally, the excipient should provide or contribute to the powder characteristics suitable for use in forming a dispersion, particularly for pulmonary administration.

In particular, the excipient should contribute to the dry powder being dispersible. By "dispersible," it is meant that the dry powder will be readily dispersible in a flowing gas stream, and further, that the individual powder particles will resist forming aggregations or otherwise agglomerating when attempts are made to form a dispersion. It has been found that nucleic acids associated with lipids are subject to aggregation/agglomeration when formed into particles by spray-drying processes. Such a tendency to form aggregations can be eliminated or greatly reduced by the proper selection of excipient(s).

Suitable excipients to be used in the compositions of the present invention include proteins, peptides, hyaluronic acid, sugars, sugar alcohols, oligosaccharides, polysaccharides, other carbohydrates, amino acids, organic salts, inorganic salts, and other polymers such as polyvinylpovidone (PVP). If lipids are used in the formulation, high molecular weight excipients, are preferred e.g., above 1 kD, usually above 3 kD, where the excipient is present at least 20% by weight of the particles, preferably at least 50% by weight, often at least 60% by weight, frequently at least 70% by weight, and sometimes 80% by weight, or more. Preferably, the excipients will be surface active to encapsulate the nucleic acid complex or construct. Preferred high molecular weight excipients include water soluble proteins, particularly serum albumins, and most particularly human serum albumin (HSA). HSA is particularly preferred as an excipient when combined with cationic lipid-containing nucleic acid complexes, where it has been found that the use of HSA excipients tends to increase both the amount of nucleic acid which may be incorporated into the particles as well as the efficiency of incorporation. In addition, HSA enhances the aerosol properties over those which can be achieved when lower molecular weight and/or non-surface active excipients are used. The use of HSA excipients in spray drying processes as described hereinafter has been found to inhibit melting and loss of the lipids from the DNA complexes during the drying process.

Optionally, the nucleic acid/excipient particles may be present in excess powdered excipient material, which may be the same or a different excipient from that which forms the matrix.

Hydrophilic excipient materials suitable for use in the compositions of the present invention will be able to form a dried matrix in which the nucleic acid complexes or constructs are dispersed in order to stabilize the nucleic acid molecules during storage, facilitate dispersion of the nucleic acids in dry powder aerosols, and enhance wetting and subsequent contact of then nucleic acids with the moist target locations within a patient or other treated host. A sufficient amount of excipient will be present to form a dry powder matrix in which the nucleic acid complexes or constructs are dispersed, typically being present in the resulting particles at a weight ratio as set forth above for the aqueous solutions. Suitable excipient materials include those listed in Table 1.

Table 1

EXCIPIENT	EXAMPLES
Proteins and Peptides	Human serum albumins, collagens; gelatins; lung surfactant proteins
Polysaccharides Mucopolysaccharides	Hyaluronic acid
Sugars	Lactose, sucrose; trehalose
Sugar alcohols	Mannitol
Oligosaccharides	Raffinose, stachyose
Other carbohydrates	Dextrans, maltodextrans, dextrins; cyclodextrins, maltodextrins, cellulose, methylcellulose, hydroxyethyl starch

EXCIPIENT	EXAMPLES
Amino acids	Glycine, alanine, glutamate
Organic salts	Lactate, tartrate, citrate salts
Inorganic salts	NaCl; $NaHCO_3$; Na_2HPO_4; NaH_2PO_4
Polymers	Polyvinylpovidone

When lipid-containing nucleic acid compositions according to the present invention are prepared by spray-drying, it will be particularly desirable to employ a high molecular weight molecule excipient which will coat, encapsulate, and/or protect the liposomes or other lipid-containing structure during the spray-drying process. Preferably, a large molecule excipient will be present at at least about 30% by weight of the solids content of the aqueous solution to be spray-dried, more preferably being present at at least 50% by weight, and still more preferably being present at at least 80% by weight. The preferred large molecule excipients are water soluble proteins or peptides, such as albumin, insulin, alpha-1-antitrypsin antibodies, most preferably being albumins, such as bovine serum albumin (BSA) and human serum albumin (HSA). In addition to the large molecule excipient, the solution to be spray-dried may include other excipients, buffers, sodium citrate, trehalose, and the like.

The dry powder formulations of the present invention may conveniently be formulated by first suspending the nucleic acid complexes and constructs in aqueous solutions of the hydrophilic excipient. The relative amounts of nucleic acid complex or construct and hydrophilic excipient material will depend on the desired final ratio of nucleic acid to excipient. Conveniently, the ratio of nucleic acid construct to excipient will be in the range from about 10:1 to 1:1000 (nucleic acid: excipient), preferably from 1:10 to 1:500 with a total solids

concentration in the aqueous suspension being usually less than 5% by weight, more usually being less than 3% by weight.

In the case of nucleic acid constructs comprising viral vectors, including adenoviral vectors, it is usually desirable that the aqueous solution be buffered in order to enhance the activity of the viral vectors after drying. Examples of buffering for the aqueous solution include the use of 130-150 mM sodium phosphate, pH 7.0, 150 mM NaCl, or the use of phosphate-buffered saline (PBS), 5% sucrose.

Preparation of the dry powder compositions of the invention can be performed by standard techniques. Such techniques include spray drying, vacuum drying, lyophilization, extrusion processes, jet milling, super critical fluid procedures and others known to those skilled in the art. Preferably, the dry powders are prepared by spray drying techniques.

Spray-drying involves processing a liquid solution into a powder. The solution to be processed is atomized into liquid droplets and is dried into solid particulates using heated air. The airborne particulate is then separated from the air using a cyclone. An example of a spray-drying apparatus is the Buchi-190 mini-spray dryer. With respect to the present invention, the complexes can be combined with an excipient and/or with bulking agents such as mannitol or glycine and/or with surface modifiers such as human serum albumin (HSA). The solution can be fed into a spray-drying apparatus which has been set for certain parameters, including feed rate, inlet and outlet temperatures, and atomizer air flow rate.

The aqueous solution can be spray-dried under conditions which result in a powder containing particles within a desired size range, typically but not necessarily having a mean particle diameter in the range from about 0.5 μm to 200 μm, with the precise particle size depending on the eventual use. For lung delivery, the particle size will typically be in the range from 0.5 μm to 10 μm, usually being from 0.5 μm to 7 μm, and preferably from 1 μm to 4 μm.

Higher total solids concentrations within the aqueous solution will generally result in larger particle sizes. Powders having an average particle size above 10 μm, usually in the range from about 20 μm to 50 μm, can be thus formed, and are particularly useful for nasal, dermal, surgical, and wound applications where it is desired that the powder rapidly settle on a target location.

Dry powders can also be prepared by vacuum drying, either at room temperature or under freezing temperatures (lyophilization). Typically, the process beings with an aqueous solution having relatively high solids content. For example, in the preparation of particles with a size of 0.5 μm to 10 μm, the liquids will usually have an initial solids content from 0.2% to 1% by weight. The vacuum drying results in a crude powder which can then be further ground, typically by jet milling, to produce a product having a uniform particle size and a desired particle size. The use of a cryoprotectant in the preparation of powders by vacuum drying is within the scope of the invention.

The preparation of a dry powder composition of the invention can be analyzed with respect to various parameters, including particle size, particle size distribution (PSD), dryness, particle cohesiveness, and mass median aerodynamic diameter (MMAD).

Particles according to the present invention will preferably have a particle size distribution of about 1 to 5 μm mass median aerodynamic diameter (MMAD), preferably from 1.5 to 4.5 μm MMAD, and more preferably from 1.5 to 4 μm MMAD. MMAD is defined as the fiftieth percentile on a mass basis in a log probability plot as determined by cascade impaction. The powdered particles will have an average particular size in the range from 0.5 μm to 200 μm, preferably being in the range from 0.5 μm to 5 μm for lung delivery, with larger sizes being useful for delivery to other moist target locations.

The aerosol particle size distribution can be measured by standard techniques, including the use of cascade impaction, such as the Andersen 1 ACFM non-viable ambient particle sizing sampler (Andersen Instrument, Smyrnia, Georgia), or by laser diffraction, such as the Malvern MasterSizer (Malvern Instruments, Inc., Southborough, Massachusetts). Both techniques can determine the aerosol particles' mass median aerodynamic diameter (MMAD). The aerodynamic diameter is defined as the diameter of a unit density sphere having the same settling speed as the particle in question of whatever shape and density. The MMAD would then be defined as that point at which half of the mass of aerosol particles are larger and half are smaller. The MMAD is typically reported in units of microns.

Powder should preferably be "dry," i.e., having a moisture content below 10% by weight, preferably below 5% by weight, and more preferably below 3% by weight.

Dispersability of a dry powder means that the dry powder will be readily dispersible in a flowing gas stream, and further, that the individual powder particles will resist forming aggregations or otherwise agglomerating when attempts are made to form a dispersion. Dispersability of a dry powder can be determined using a dry powder inhaler or a test bed. For example, a blister pack filled with powder is inserted into the device. The aerosol produced in the device chamber is drawn at a defined suction flowrate and then collected on a membrane filter. Dispersability is the fraction of powder mass which is collected on the filter relative to the mass which is inserted into the blister pack.

A further advantage of a dry powder composition is the ability to easily store such formulations until the need for use arises. Dry powders thus prepared according to the invention can be stored at less than 10% relative humidity until the need for dispersion in a dry powder device for inhalation.

As previously described, the cationic complexes of polymer-modified (*e.g.*, PEGylated) adenovirus exhibit heightened levels of infectivity in both immune subjects and naive (*i.e.*, non-immune) subjects. However, PEGylation of the virus exceeding 15% can cause a decrease or ablation of viral infectivity, thereby providing a disincentive for further PEGylation. In addition, decreases in viral infectivity can occur at levels less than 15% (*e.g.*, 10%) depending on the glycol polymer selected for attachment (*e.g.*, TMPEG vs. MPEG). Accordingly, the cationic complexes of the present invention provide a solution to this problem by allowing significantly greater levels of polymer modification to be used (*e.g.*, 20% or greater) while maintaining viral infectivity levels comparable to unmodified (*i.e.*, non-polymer-modified) adenovirus. In fact, as demonstrated in the examples set forth below, the complexes of the present invention provide infectivity levels significantly greater that either unmodified adenovirus and polymer-modified adenovirus.

Infectivity of the adenoviral vector complexes of present invention are assessed by standard infection assays. For example, the ability of adenovirus to infect a cell is assessed by monitoring the expression of a transgene (*e.g.*, a reporter gene such as lacZ) contained within the adenovirus. Genetic reporter systems are well-known in the art, and are disclosed for example in Short Protocols in Molecular Biology, 1995, Ausubel et al., eds., 3rd edition, Wiley

and Sons, Inc., the disclosure of which is incorporated by reference. The adenoviral vector is engineered by standard recombinant DNA methods to contain a transgene, and the complexed adenoviral vector is used to infect cells that are permissive for the virus. After infection under standard conditions, cell lysates are analyzed for the presence of the product of the transgene, *e.g.*, β-galactosidase. For example, the product of the transgene can be assessed by colorimetric, chemiluminescence or fluorescence assays, or immunoassays. In this way, those of ordinary skill in the art can compare complexed and uncomplexed adenoviral vector, and can determine the optimal percentages and conditions for glycolization and cationic complexing that result in optimum retention of infectivity.

Alternatively, if the transgene encodes CFTR, infectivity can be measured by acsertaining the ability of the CFTR protein expressed in cultured CF airway epithelia to correct the Cl^- channel defect following the methods described by Rich et al., Nature 347: 358, 1990, incorporated herein by reference. Briefly, cultured CF airway epithelial cells are infected with adenoviral vectors containing DNA encoding a CFTR protein. Virus-mediated expression of functional CFTR protein is assessed using an SPQ [6-methoxy-N-(3-sulfopropyl)-quinolinium, Molecular Probes, Eugene, OR] halide efflux assay. SPQ is a halide-sensitive fluorophore, the fluorescence of which is quenched by halides. In this assay, cells are loaded with SPQ, CFTR is activated by cAMP agonists, the CFTR Cl^- channel opens, halides exit the cell, and SPQ fluorescence in the cell increases rapidly. Thus increases in intracellular fluorescence in response to cAMP provide a measure of a functional Cl^- channel.

In another assay suitable for measuring viral infectivity, CF epithelial cells are infected with adenoviral vectors containing DNA encoding a CFTR protein, and secretion of Cl^- from infected cells is measured in response to cAMP stimulation. The secretion of Cl^- can be measured as an increase in transepithelial short-circuit current with addition of cAMP agonists, as described for example by Rich et al., Human Gene Therapy 4: 461, 1993, the disclosure of which is incorporated herein by reference. Expression of a functional CFTR protein can also be assessed by patch clamp techniques that detect reversibly activated whole-cell currents in response to addition of cAMP agonists, or single-channel currents in excised, cell-free patches of membrane in response to cAMP-dependent protein kinase and ATP. Patch clamp techniques are

described for example by Sheppard et al., Cell 76: 1091, 1994, and U.S. Patent No. 5,639,661, the disclosures of which are incorporated herein by reference.

Retention of infectivity is defined herein as an infectivity level sufficient to have therapeutic value, for example at least about 20% infective relative to unmodified virus (non-complexed, non-polymer-modified adenovirus). For some embodiments, the virus complex maintains at least 60% infectivity. In other therapeutic embodiments, the complexed modified virus is preferred to maintain at least 80% infectivity. Lower percent infectivity of at least 5% may be useful for applications such as viral oncolysis.

In a particular example of an infectivity assay, an adenoviral vector containing the β-galactosidase (β-gal) reporter gene (lacZ) is covalently modified by exposure to various concentrations of TMPEG and subsequently complexed with a cationic molecule, other than poly-L-lysine (*e.g.*, DEAE-dextran). A cell line that supports adenoviral vector propagation, for example, 293 or 3T3 cells, is exposed to unmodified and modified/complexed adenoviral vector containing the β-gal gene. Cells are then incubated under conditions appropriate for β-gal expression. The presence of β-gal in cell lysates is measured by standard colorimetric, fluorescence, or chemiluminescence assays, *e.g.*, by using X-gal. The quantity of β-gal in 293 cell lysates provides a measurement of the ability of the complexed, PEGylated adenovirus to infect 293 cells. The complexed, PEGylated virus that maintains 50% infectivity relative to unmodified virus is considered to retain infectivity.

The complexed, polymer-modified adenoviruses of the present invention exhibit reduced antigenicity relative to unmodified virus. Reduced antigenicity is defined as a statistically significant ($p>0.05$) reduction in binding of the polymer-modified virus to neutralizing antibodies against the virus. Reduced antigenicity is assessed by methods known in the art, including *in vitro* and *in vivo* assays. For example, both modified and unmodified viruses containing reporter genes are incubated in the presence or absence of neutralizing antibodies or serum. The antibody-treated viruses and non-antibody treated control viruses are then used to infect cells as described above, and reporter gene expression in infected cells is performed as described above. With unmodified viruses, treatment with neutralizing antibodies results in lower levels of infection and thus lower levels of transgene expression. The

complexed, polymer-modified adenoviruses of the present invention are protected from neutralization by the polymer coating, and thus provide increased infectivity and increased transgene expression in the present assays relative to unmodified viruses that have been exposed to neutralizing antibodies, a particular advantage of the invention.

By utilizing the foregoing assays, those of ordinary skill in the art can determine the conditions for glycolization and subsequent complexing necessary to provide a complexed, polymer-modified adenovirus that maintains infectivity and exhibits reduced antigenicity. The dry powder compositions can also be tested in animal models. For example, rodents can be used in testing the aerosolized compositions of the invention with the use of a whole-body exposure chamber.

Because of their unique properties, the dry powder compositions containing the adenoviral vector complexes of the invention have particular utility for the in vivo delivery of a transgene to target cells or tissues. Aerosolization of the compositions allows for deep penetration of the adenoviral vector complexes into target tissue, especially advantageous where the target is the lower respiratory tract. An additional advantage is that such compositions, when administered with a device such as dry powder inhaler, allow an individual in need of such a composition to self-administer when necessary. These compositions further provide adenoviral vectors with reduced antigenicity and/or immunogenicity, allowing for repeat administration of the adenoviral vector complexes. In a particular embodiment of the invention, dry powder formulations of adenoviral vector complexes containing the EPO transgene have particular utility and are advantageous as they are readily administered and provide transient expression of EPO for such clinical indications as increasing pre-surgical hematocrit levels or the treatment of anemia. In other embodiments of the invention, dry powder formulations of adenoviral vector complexes containing the transgene for G-CSF or GM-CSF have utility for the provision of proteins which can increase blood neutrophil concentration in various clinical settings, for example, administration to an individual following chemotherapy.

The complexes of the present invention also have utility in medical therapy and diagnosis in medical and veterinary practice and in agriculture. The complexed, polymer-modified adenoviruses are particularly useful for delivering a transgene to a target cell for the

treatment of various disorders, for example in which the transgene product is absent, insufficient, or nonfunctional. They are of particular use in the transfer of genes, toxins and/or diagnostic markers. Alternatively, the expression of the transgene may serve to block the expression or function of an undesired gene or gene product in the target cell. An additional application is in the creation of tolerogens for viral antigens.

The invention is also directed to methods for introducing a transgene into a target cell. The methods comprise administering a dry powder composition to an individual which allows a complexed, polymer-modified adenovirus of the present invention to be delivered to a target cell for expression of a transgene by inhalation or the use of any delivery device known to those skilled in the art.

Target cells for adenovirus complexes of the present invention are any cell in which expression of a transgene is desired. Target cells include cell types permissive to adenovirus infection (*e.g.*, 293 cells and A549 cells) and cell types resistant to adenovirus infection (*e.g.*, human epithelial cells, NIH 3T3 cells, and 9L gliosarcoma cells). In fact, the complexed, polymer-modified adenoviral vectors are particularly suitable for infecting adenovirus resistant cells for transgene expression. While not wishing to be bound by theory, the complexes of the present invention do not require binding to the Coxsackie Adenovirus Receptor (CAR) for internalization. As may be apparent to the skilled artisan, internalization of adenovirus in permissive cell types is generally dependent on the CAR pathway. However, the complexes of the present are internalized by pathways other than CAR, which renders them particularly suitable for transgene expression in adenovirus resistant cell types (Fasbender et al., J. Biol. Chem. 272:6479-6489, 1997; Kaplan et al., Hum. Gen. Ther. 9:1469-1479, 1998), the disclosure of which are incorporated by reference.

The dry powder compositions of the invention can be administered to the lungs of an individual using dry powder inhalers that utilize the individual's inspiratory force to disperse and deliver the powder. Such an inhalers may alternatively use its own source of compressed gas or an auxiliary source of gas to disperse and deliver the powder. Alternatively, the powder can be delivered by delivery devices that can disperse the powder by electrostatic forces. Preferably, the dry powder compositions are delivered to an individual in doses ranging from 2-

10 milligrams. Successive administration of such doses could given to an individual until a desired amount of viral vector was delivered to the lung.

The compositions of the invention are provided in aerosol form, and such compositions may be provided as a dry powder. The advantage of such formulations is the ease of delivery to an individual, expecially with respect to the lung, where such formulation may be sprayed or inhaled into the target tissues for uptake. Aerosolized compositions may contain adenovirus or naked DNA alone, adenovirus complexed with polymers or complexed with cationic molecules or adenovirus complexed with polymers and cationic molecules. The adenoviruses used in the complexes may be any one or more adenoviruses of choice which have been engineered to contain one or more transgenes for delivery to the individual. The adenovirus is delivered as a composition in combination with a physiologically acceptable carrier. As used herein, the term "physiologically acceptable carrier" includes any and all solvents, diluents, isotonic agents, and the like.

The formulation of compositions is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, PA. The complexed, polymer-modified adenoviruses are compounded for convenient and effective administration.

The effective amounts of the complexed, polymer-modified adenovirus to be used in accordance with the present invention for humans, or any other mammal, can be further optimized by the ordinary skilled artisan with consideration of individual differences in age, weight and condition of the subject and the particular transgene(s) to be delivered. Consideration can also be given as to whether the adenoviral vector complexes are to be administered in a series of doses to provide bursts of transient expression of a transgene.

It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated, each unit containing a predetermined quantity of active material calculated to produce the desired effect in association with the required carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly depend on the unique characteristics of the polymer-

modified viruses and the limitations inherent in the art of compounding and preparation of dry powder compositions. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the ingredients.

The practice of the invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular virology, microbiology, recombinant DNA technology and pharmacology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley and Sons, Inc., New York, 1995, Remington's Phartmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, PA 1985, and Encyclopedia of Chemical Technology, 4th ed., Kirk-Othmer, ed., John Wiley and Sons, Inc., 1991.

CLAIMS

WE CLAIM:

1. A dry powder composition comprising a complex of a cationic molecule and of an adenoviral vector having at least one polyalkalene glycol polymer bound thereto.

2. An aerosol composition comprising a complex of a cationic molecule and of an adenoviral vector having at least one polyalkalene glycol polymer bound thereto.

3. The composition of Claim 1, in which the polyalkalene glycol polymer is polyethylene glycol.

4. The composition of Claim 2, in which the polyalkalene glycol polymer is polyethylene glycol.

5. The composition of Claim 3, in which the cationic molecule is DEAE-dextran.

6. The composition of Claim 4, in which the cationic molecule is DEAE-dextran.

7. The composition of Claim 5, in which the adenoviral vector contains a transgene encoding cystic fibrosis transmembrane regulator operably linked to expression control sequences.

8. The composition of Claim 6, in which the adenoviral vector contains a transgene encoding cystic fibrosis transmembrane regulator operably linked to expression control sequences.

9. The composition of Claim 5, in which the adenoviral vector contains a transgene encoding erythropoietin operably linked to expression control sequences.

10. The composition of Claim 6, in which the adenoviral vector contains a transgene encoding erythropoietin operably linked to expression control sequences.

11. A method for providing transgene expression to an individual, comprising administering a dry powder composition comprising a complex of a cationic molecule and of an adenoviral vector having at least one polyalkalene glycol polymer bound thereto, to said individual.

12. A method for providing transgene expression to an individual, comprising administering an aerosol composition comprising a complex of a cationic molecule and of an adenoviral vector having at least one polyalkalene glycol polymer bound thereto, to said individual.

INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/US 99/28633

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US ROMANCZUK H ET AL: "Modification of an adenoviral vector with biologically selected peptides: a novel strategy for gene delivery to cells of choice 'see comments!." retrieved from STN Database accession no. 2000031165 XP002133225 abstract & HUMAN GENE THERAPY, (1999 NOV 1) 10 (16) 2615-26. , ---	1-12
Y	WO 96 32116 A (INHALE THERAPEUTIC SYST) 17 October 1996 (1996-10-17) claims 1,4,7 --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 March 2000	28/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Berte, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/US 99/28633

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 39467 A (HENERSON DANIEL R ;LAMPARSKI HENRY G (US); CALYDON INC (US); SCHIUI) 11 September 1998 (1998-09-11) claims 1,33-39	1-12
P,A	WO 98 56363 A (BYRNE BARRY ;LEONG KAM W (US); MAO HAI QUAN (US); WANG YAN (US); U) 17 December 1998 (1998-12-17) claims	1-12
P,Y	MARLOW S.A. ET AL: "viraMASC(TM): A biologically optimised pegylation technology to target adenovirus to tumours." PROCEEDINGS OF THE CONTROLLED RELEASE SOCIETY, (1999) -/26 (555-556). , XP002133224 figure 1	1-12
E	WO 00 11202 A (GENZYME CORPORATION, USA) 2 March 2000 (2000-03-02) claims; examples 1,2	1-12
Y	WO 96 17948 A (UNIV MICHIGAN) 13 June 1996 (1996-06-13) claims 1,8,9	1-12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/28633

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 11-12
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 11-12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/US 99/28633

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9632116 A	17-10-1996	US 5994314 A	30-11-1999
		AU 5743996 A	30-10-1996
WO 9839467 A	11-09-1998	AU 6187698 A	22-09-1998
		AU 6345098 A	22-09-1998
		AU 6445698 A	22-09-1998
		EP 0968298 A	05-01-2000
		WO 9839464 A	11-09-1998
		WO 9839466 A	11-09-1998
WO 9856363 A	17-12-1998	AU 7836598 A	30-12-1998
WO 0011202 02 A		NONE	
WO 9617948 A	13-06-1996	US 5811274 A	22-09-1998
		CA 2206600 A	13-06-1996
		EP 0797680 A	01-10-1997
		JP 10510156 T	06-10-1998
		US 5654185 A	05-08-1997
		US 5804431 A	08-09-1998